

# Sensitization for Anticancer Drug–Induced Apoptosis by Betulinic Acid<sup>1</sup>

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## Abstract

We previously described that betulinic acid (BetA), a naturally occurring pentacyclic triterpenoid, induces apoptosis in tumor cells through the mitochondrial pathway. Here, for the first time, we provide evidence that BetA cooperated with anticancer drugs to induce apoptosis and to inhibit clonogenic survival of tumor cells. Combined treatment with BetA and anticancer drugs acted in concert to induce loss of mitochondrial membrane potential and the release of cytochrome *c* and Smac from mitochondria, resulting in activation of caspases and apoptosis. Overexpression of Bcl-2, which blocked mitochondrial perturbations, also inhibited the cooperative effect of BetA and anticancer drugs, indicating that cooperative interaction involved the mitochondrial pathway. Notably, cooperation of BetA and anticancer drugs was found for various cytotoxic compounds with different modes of action (e.g., doxorubicin, cisplatin, Taxol, VP16, or actinomycin D). Importantly, BetA and anticancer drugs cooperated to induce apoptosis in different tumor cell lines, including *p53* mutant cells, and also in primary tumor cells, but not in human fibroblasts indicating some tumor specificity. These findings indicate that using BetA as sensitizer in chemotherapy-based combination regimens may be a novel strategy to enhance the efficacy of anticancer therapy, which warrants further investigation.

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**Keywords:** Apoptosis, betulinic acid, cancer, mitochondria, resistance.

in activation of effector caspases [4]. The mitochondrial pathway plays a crucial role in drug-induced apoptosis [5]. On induction of apoptosis, apoptogenic factors such as cytochrome *c*, apoptosis-inducing factor (AIF), or second mitochondria-derived activator of caspase (Smac)/DIABLO are released from mitochondria into the cytosol [6]. Cytochrome *c* triggers caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex, whereas Smac/DIABLO promotes caspase activation by neutralizing the inhibitory effects to inhibitor of apoptosis protein (IAP) [6].

Because most antitumor therapies including chemotherapy primarily act by inducing apoptosis in cancer cells, defects in apoptosis programs may cause resistance [2,7]. Combination treatment may prove to be advantageous in malignancies that still partially respond to either treatment alone because they may help to amplify weaker death signals. Thus, combination therapies may constitute a potent strategy for bypassing resistance. Betulinic acid (BetA) is a naturally occurring pentacyclic triterpenoid derived from white birch trees, which induces apoptosis in a variety of tumor cells [8–10]. We previously found that BetA-induced apoptosis differs from anticancer agents such as doxorubicin because BetA-induced apoptosis is not associated with activation of ligand/receptor systems such as CD95 and does not involve *p53* [11]. In BetA-induced apoptosis, perturbation of mitochondrial function, including loss of mitochondrial permeability transition, precedes other key features of apoptosis such as activation of the caspase cascade and nuclear fragmentation [12]. In search for novel strategies to enhance the efficacy of anticancer therapy, we asked whether or not triggering the mitochondrial pathway by BetA would modulate the sensitivity of tumor

## Introduction

Primary or acquired resistance of many tumors to established treatment regimens still constitutes a major concern in oncology [1]. Thus, attempts to improve the survival of cancer patients largely depend on strategies to target tumor cell resistance. Induction of apoptosis in cancer cells is a key killing mechanism for most antitumor therapies including chemotherapy,  $\gamma$ -irradiation, immunotherapy, or cytokines [2,3]. Apoptosis pathways may be initiated through different entry sites, such as death receptors (receptor pathway) or mitochondria (mitochondrial pathway), resulting

Abbreviations: BetA, betulinic acid; CMXRos, chloromethyl-X-rosamine; FACS, fluorescence-activated cell sorting; IAP, inhibitor of apoptosis protein; Smac, second mitochondria-derived activator of caspase; TRAIL, TNF-related apoptosis-inducing ligand; zVAD.fmk, benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone

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cells for anticancer drug-induced apoptosis. Here, for the first time, we provide evidence that combined treatment with BetA and anticancer drugs cooperated to induce apoptosis in tumor cells.

## Materials and Methods

### Cell Culture

SHEP neuroblastoma cells, SHEP neuroblastoma cells transfected with vector control (Neo) or Bcl-2, medulloblastoma (Daoy, *p53* mutant), glioblastoma (A172), melanoma (Mel-Juso) cells, or primary renal cell carcinoma cells obtained from a patient with metastatic renal cell carcinoma were maintained in RPMI 1640 or DMEM medium (Life Technologies, Inc., Eggenstein, Germany) as previously described [13]. Human fibroblasts (PromoCell, Heidelberg, Germany) were maintained according to the manufacturer's instructions. A total of  $0.5 \times 10^5$  cells/ml were cultured in 96-well, 24-well, or 6-well plates or in 75-cm<sup>2</sup> flasks (Falcon, Heidelberg, Germany).

### Determination of Apoptosis, Cell Viability, and Clonogenic Growth

Cells were incubated with BetA (BioService Halle GmbH, Halle, Germany), doxorubicin, VP16, cisplatin, Taxol (all from Sigma, Deisenhofen, Germany), or benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk; Bachem, Heidelberg, Germany) at indicated concentrations and times. Apoptosis of adherent and detached cells was assessed by fluorescence-activated cell sorting (FACS) analysis of DNA fragmentation of propidium iodide-stained nuclei in permeabilized cells as previously described [13,14]. Cell viability was determined by MTT assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Clonogenic growth was assessed by crystal violet staining as previously described [15].

### Western Blot Analysis

Western blot analyses were performed as previously described [15], using mouse anti-caspase-8 monoclonal antibody C15 (1:10 dilution of hybridoma supernatant; kindly provided by P. Krammer), mouse anti-caspase-3 monoclonal antibody (1:1000; BD Biosciences, Heidelberg, Germany), mouse anti-XIAP monoclonal antibody (1:1000, H62120; BD Biosciences), mouse anti-cytochrome *c* monoclonal antibody (1:1000; BD Biosciences), rabbit anti-Smac polyclonal antibody (1:1000; Calbiochem, Bad Soden, Germany), and  $\beta$ -actin monoclonal antibody (1:10000; Sigma), followed by goat antimouse IgG or goat antirabbit IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). Enhanced chemiluminescence (ECL; Amersham Pharmacia, Freiburg, Germany) was used for detection. Expression of  $\beta$ -actin was used to control for equal gel loading.

### Assessment of Caspase Activity

Caspase activity was determined by fluorogenic substrates for caspase-3, caspase-8, or caspase-9 according

to the manufacturer's instructions (caspase-3, Roche; caspase-8, BioCat GmbH, Palo Alto, CA; caspase-9, Bachem). Caspase activity was measured fluorometrically at 535 or 505 nm on a microplate fluorescence reader (1420 Victor Multilabel Counter; Wallac, Rodgau-Jugesheim, Germany).

### Preparation of Mitochondria or Cytosolic Extracts

Preparation of mitochondria or cytosolic extracts was performed using the ApoAlert cell fractionation kit (BD Biosciences) according to the manufacturer's instructions.

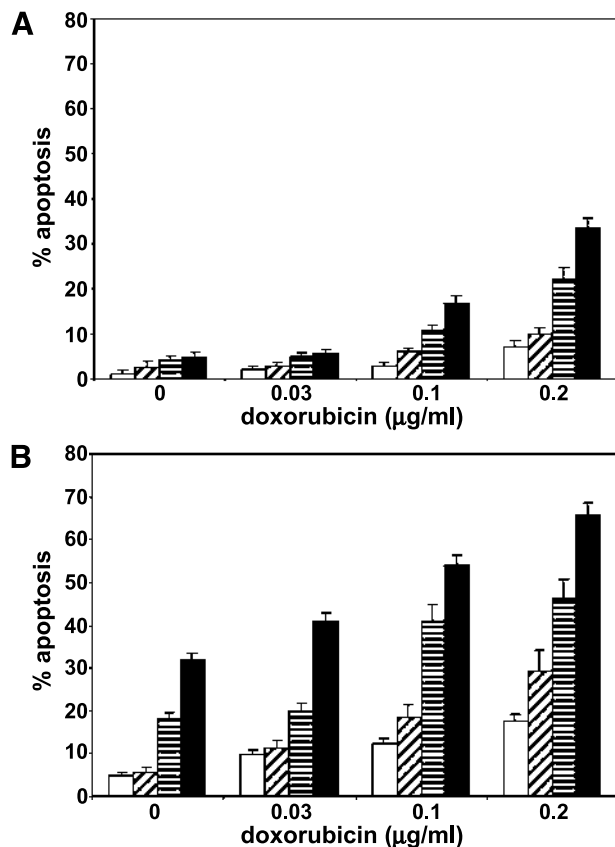
### Assessment of Mitochondrial Transmembrane Potential or Cytochrome *c* Release

The cationic lipophilic fluorochrome chloromethyl-X-rosamin (CMXRos) (1  $\mu$ M; Molecular Probes, Eugene, OR) was used to measure the mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) [16]. Cells were incubated for 15 minutes at 37°C in the presence of fluorochrome, washed in PBS/1% fetal calf serum (FCS), and immediately analyzed by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany). CMXRos was recorded in fluorescence 3. Cytochrome *c* release was determined in permeabilized cells using anti-cytochrome *c* monoclonal antibody (BD Biosciences) as previously described [17].

## Results

### BetA and Anticancer Drugs Cooperate to Induce Apoptosis in Tumor Cells

In search for novel strategies to enhance the efficacy of chemotherapy, we investigated the effect of the mitochondriotropic natural compound, BetA, on anticancer drug-induced apoptosis. For these studies, we selected SHEP neuroblastoma cells, which have previously been proven suitable for studies on apoptosis pathways by our group and other investigators [11,18]. Treatment of SHEP neuroblastoma cells with relatively low doses of either BetA or doxorubicin alone induced only minimal apoptosis (Figure 1, A and B). Interestingly, the combined treatment with BetA, together with doxorubicin, cooperated to induce apoptosis in SHEP neuroblastoma cells in a dose- and time-dependent manner, as determined by analysis of DNA fragmentation (Figure 1, A and B). Similar results were obtained when apoptosis was assessed by Annexin V staining (data not shown). To see whether the combination treatment with BetA and doxorubicin simply delayed cell death or inhibited clonogenic survival of tumor cells, we performed clonogenic assays. Importantly, BetA cooperated with doxorubicin to reduce clonogenic survival of tumor cells (Figure 2). To exclude that the sensitization effect of BetA was restricted to doxorubicin, we tested BetA in combinations of various anticancer drugs with different modes of action. Also, BetA sensitized SHEP neuroblastoma cells for apoptosis in response to treatment with VP16, Taxol, or actinomycin D (Figure 3). We then extended our studies to additional human cancer cell lines, which were



**Figure 1.** Cooperation of BetA and doxorubicin to induce apoptosis. SHEP neuroblastoma cells were treated for 24 hours (A) or 48 hours (B) with BetA (0 μg/ml: white bars; 3 μg/ml: vertically hatched bars; 4 μg/ml: horizontally hatched bars; 5 μg/ml: black bars) and/or 0 to 0.2 μg/ml doxorubicin. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments.

previously found to respond to BetA [8,11], to exclude that the cooperative effect of BetA and anticancer drugs was restricted to a particular cell line. Importantly, BetA sensitized medulloblastoma, glioblastoma, or melanoma cells for anticancer drug-induced apoptosis (Figure 4A). Furthermore, we studied the effect of BetA on primary tumor cells derived from metastatic renal cell carcinoma. Interestingly, we found that BetA sensitized primary renal cell carcinoma cells for doxorubicin- or cisplatin-induced apoptosis (Figure 4B). In contrast, BetA failed to sensitize untransformed human fibroblasts for doxorubicin-induced apoptosis indicating some tumor specificity (Figure 4A). Together, this set of experiments indicates that BetA cooperated with anticancer drugs to trigger apoptosis in different human tumor cell lines and primary tumor cells.

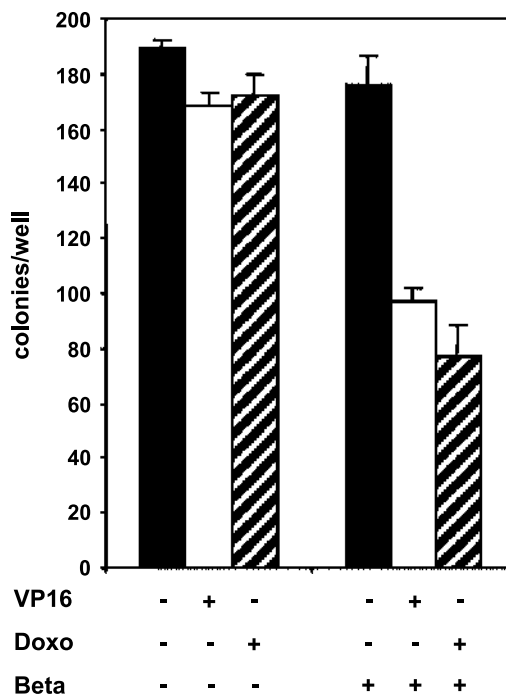
#### Beta and Anticancer Drugs Cooperate to Activate Caspases

To see whether apoptosis on treatment with BetA and anticancer drugs required caspase activation, we used the broad-range caspase inhibitor, zVAD.fmk. Apoptosis in response to treatment with BetA and doxorubicin or VP16 was almost completely blocked in the presence of zVAD.fmk,

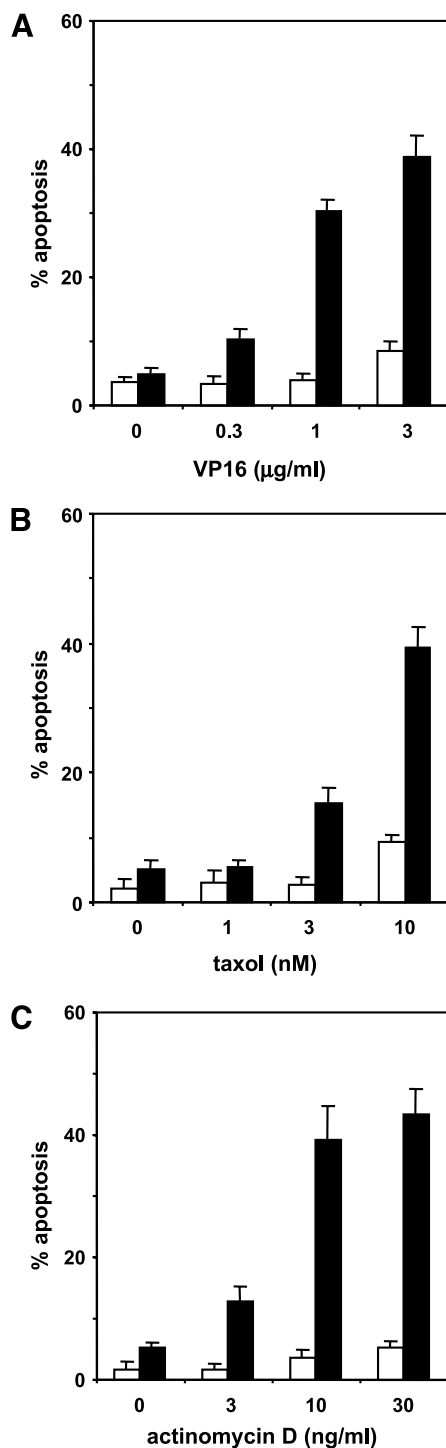
demonstrating that apoptosis was mediated by activation of caspases (Figure 5A). We then monitored caspase activation by Western blot analysis to see whether and at what level of the caspase cascade BetA- and cytotoxic drug-induced signaling pathways converged. Notably, the combined treatment with BetA and doxorubicin cooperated to activate caspase-8, caspase-3, and PARP (Figure 5B), in line with our previous findings that BetA triggers activation of caspase-3 or caspase-8 downstream of mitochondria [12]. Also, cleavage—and thus inactivation—of XIAP, an endogenous caspase inhibitor, was only observed on combination treatment with BetA and doxorubicin (Figure 5B). To directly assess caspase activity, we also performed an enzymatic caspase assay using fluorogenic caspase substrates. Cotreatment with BetA and doxorubicin or VP16 resulted in increased activity of caspase-3, caspase-9, and caspase-8 compared to either treatment alone (Figure 6). Together, this set of experiments demonstrates that BetA and anticancer drugs cooperated to activate caspases.

#### Beta and Anticancer Drugs Cooperate to Perturb Mitochondrial Functions

We next analyzed the effect of BetA and anticancer drugs on mitochondrial function because we previously found that BetA triggers mitochondrial perturbations [11]. Combined treatment with BetA and doxorubicin or VP16



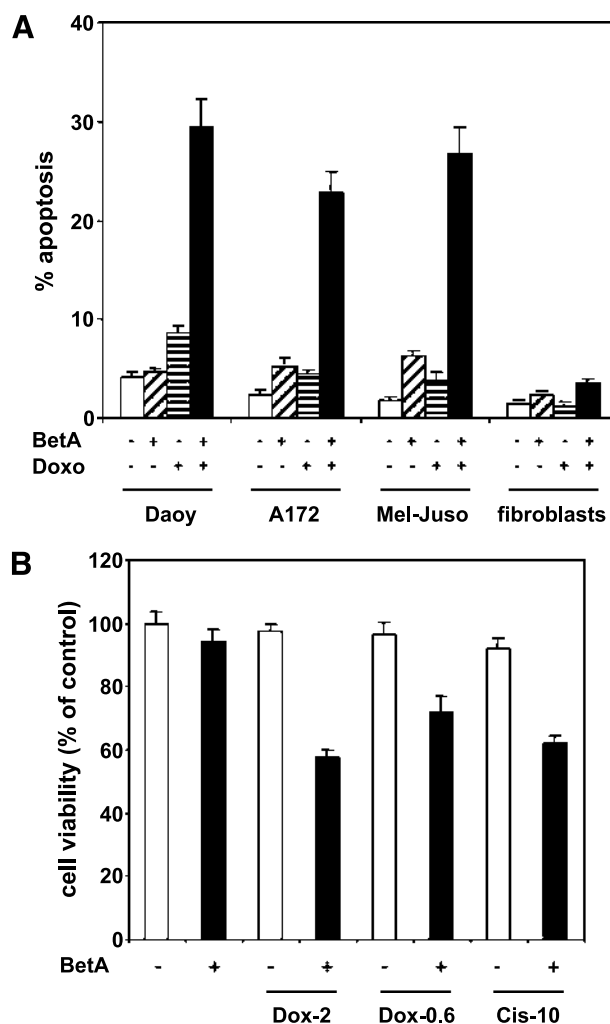
**Figure 2.** Cooperation of BetA and anticancer drugs to inhibit clonogenic survival. SHEP neuroblastoma cells were treated with 0.1 μg/ml VP16 (white bars) or 0.01 μg/ml doxorubicin (hatched bars) and/or 3 μg/ml BetA. Clonogenic survival was assessed by crystal violet staining after 7 days, and colony numbers per well of a 24-well plate are indicated. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments.



**Figure 3.** Cooperation of BetA and anticancer drugs to induce apoptosis. SHEP neuroblastoma cells were treated for 24 hours with 0 to 3  $\mu\text{g/ml}$  VP16 (A), 0 to 10 nM Taxol (B), or 0 to 10 ng/ml actinomycin D (C) in the presence (black bars) or absence (white bars) of 5  $\mu\text{g/ml}$  BetA. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments.

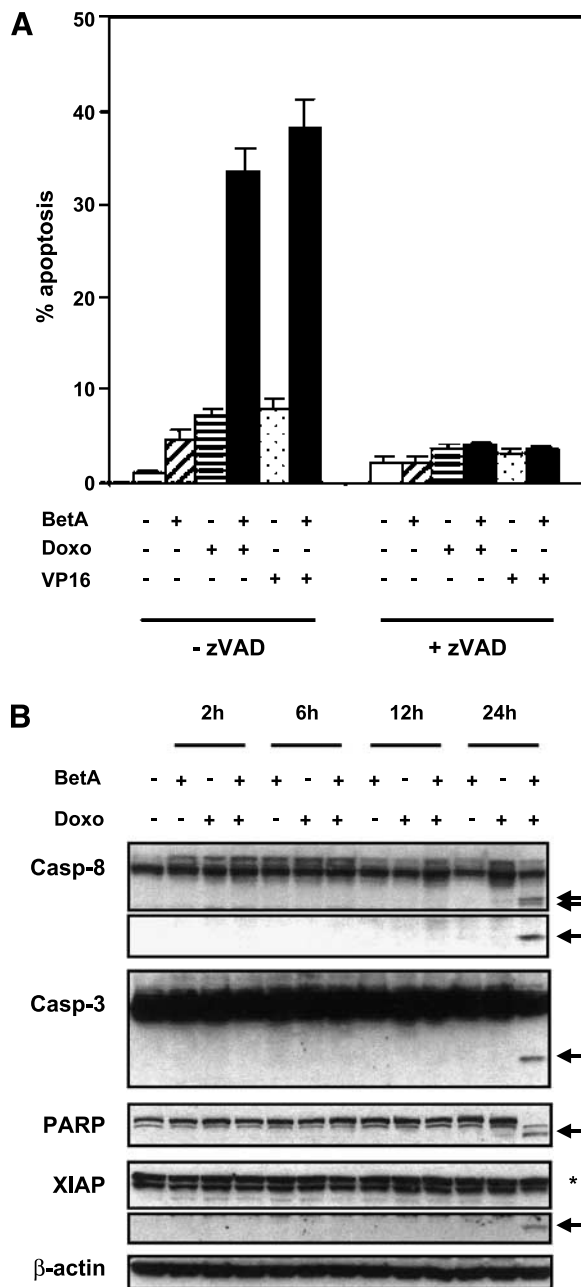
resulted in enhanced breakdown of mitochondrial membrane potential (MMP) compared to either treatment alone (Figure 7). To further address the role of mitochondria in BetA- and anticancer drug-induced apoptosis, we deter-

mined the release of cytochrome *c* from mitochondria by flow cytometry, staining cytochrome *c* in permeabilized cells. BetA and doxorubicin or VP16 acted in concert to trigger cytochrome *c* release from mitochondria compared to either treatment alone (Figure 8). Interestingly, loss of MMP induced by combined treatment with BetA and doxorubicin or VP16 preceded release of cytochrome *c* from mitochondria. In addition, we analyzed the translocation of mitochondrial proteins into the cytosol by Western blot analysis of mitochondrial and cytosolic cell fractions.



**Figure 4.** Cooperation of BetA and doxorubicin to induce apoptosis in human tumor cell lines and primary tumor cells. (A) Cooperation of BetA and doxorubicin to induce apoptosis in human tumor cell lines. Daoy medulloblastoma, A172 glioblastoma, Mel-Juso melanoma cells, or human fibroblasts were treated for 24 hours with 5  $\mu\text{g/ml}$  BetA and/or 0.2  $\mu\text{g/ml}$  doxorubicin. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments. (B) Cooperation of BetA and anticancer drugs to induce apoptosis in primary renal cell carcinoma cells. Primary renal cell carcinoma cells were treated for 4 days with 2  $\mu\text{g/ml}$  doxorubicin (Dox-2), 0.6  $\mu\text{g/ml}$  doxorubicin (Dox-0.6), or 10  $\mu\text{g/ml}$  cisplatin (Cis-10) in the presence (black bars) or absence (white bars) of 5  $\mu\text{g/ml}$  BetA. Cell survival was assessed by MTT assay. Percentage of cell viability was determined as: (survival of treated cells  $\times$  100 / survival of untreated cells). Mean and SD of triplicates are shown; similar results were obtained in two independent experiments.





**Figure 5.** Cooperation of BetA and anticancer drugs to activate caspases. (A) Effect of caspase inhibition on BetA and anticancer drug-induced apoptosis. SHEP neuroblastoma cells were treated for 24 hours with 5  $\mu$ g/ml BetA and/or 0.2  $\mu$ g/ml doxorubicin and/or 3  $\mu$ g/ml VP16 in the presence or absence of 50  $\mu$ M zVAD.fmk. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments. (B) Cooperation of BetA and doxorubicin to activate caspases. SHEP neuroblastoma cells were treated for 2 to 24 hours with 5  $\mu$ g/ml BetA and/or 0.2  $\mu$ g/ml doxorubicin. Expression of caspase-3, caspase-8, PARP, XIAP, and  $\beta$ -actin was assessed by Western blot analysis. Cleavage products are indicated by arrows and nonspecific immune-reactive bands are indicated by asterisk. A representative experiment out of three independent experiments is shown.

Increased release of cytochrome *c* and Smac from mitochondria into the cytosol was observed in cells treated with BetA and doxorubicin compared to cells treated with BetA or doxorubicin alone (Figure 9). To test whether loss of

MMP and cytochrome *c* release on treatment with BetA and anticancer drugs required caspase activity, we used the broad-range caspase inhibitor, zVAD.fmk. Interestingly, loss of MMP and cytochrome *c* release following treatment with BetA alone or with the combination of BetA and doxorubicin or VP16, were markedly reduced in the presence of zVAD.fmk (Figure 10). This indicates that loss of MMP and cytochrome *c* release on combined treatment with BetA and anticancer drugs involved caspase-dependent and also caspase-independent mechanisms.

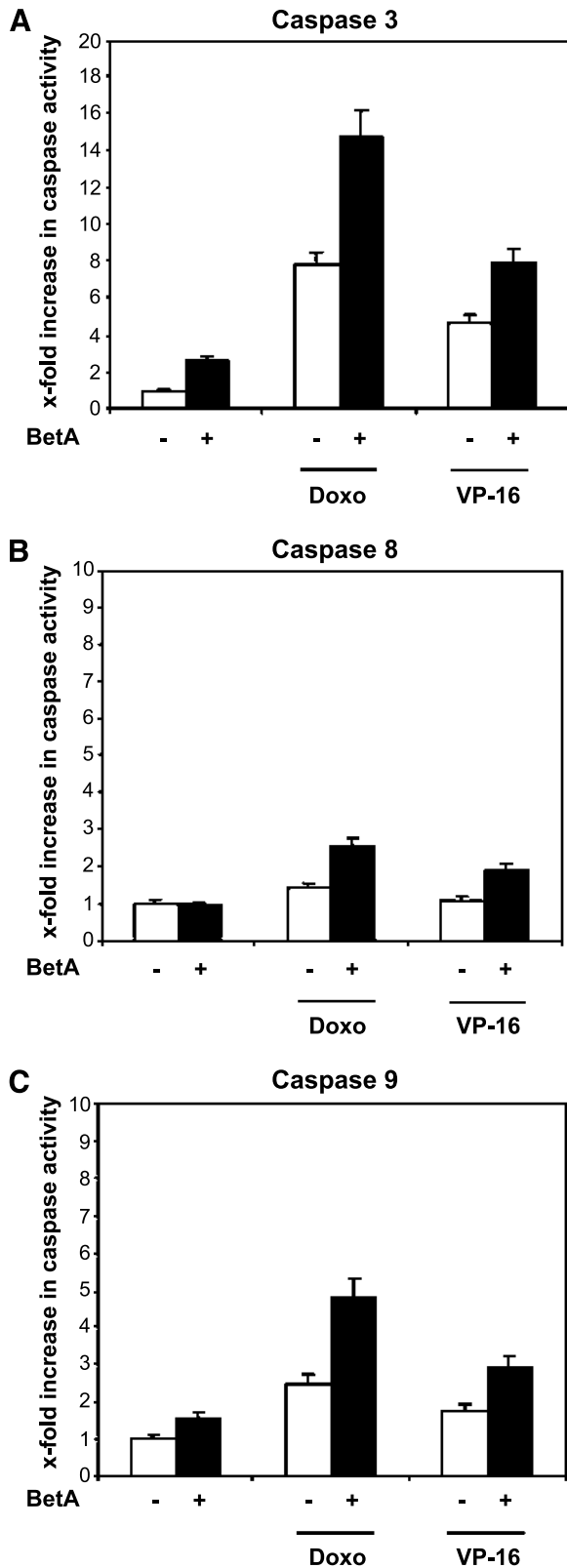
#### Overexpression of Bcl-2 Inhibits Apoptosis Induced by BetA and Anticancer Drugs

To further investigate whether the cooperative effect of BetA and anticancer drugs was mediated by mitochondrial perturbations, we used SHEP neuroblastoma cells in which the mitochondrial pathway was blocked by Bcl-2 overexpression. Overexpression of Bcl-2 inhibited loss of MMP following treatment with BetA, with doxorubicin or VP16, or with the combination of BetA and doxorubicin or VP16 (Figure 11). Also, overexpression of Bcl-2 inhibited the release of cytochrome *c* from mitochondria upon treatment with BetA and/or doxorubicin or VP16 (Figure 11). Importantly, overexpression of Bcl-2 also inhibited apoptosis in response to BetA and doxorubicin (Figure 12). This indicates that the cooperative effect of BetA and anticancer drugs was mediated through the mitochondrial pathway.

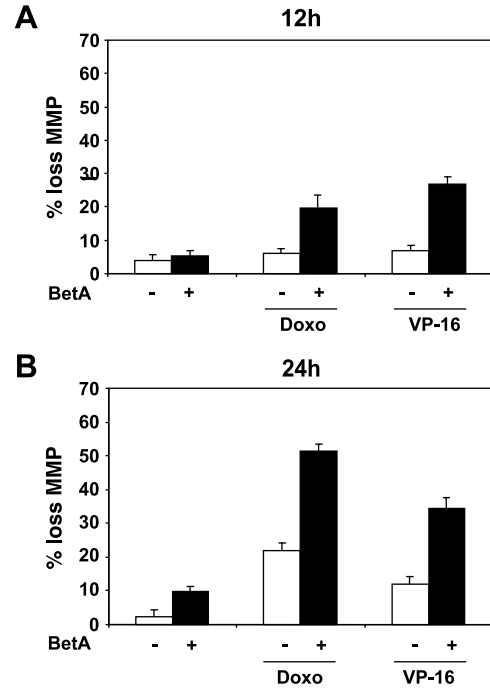
#### Discussion

Because resistance of many tumors to established treatment regimens still constitutes a major problem in cancer therapy, novel strategies to target tumor cell resistance are essential to improve patient outcome [1]. Most antitumor therapies including chemotherapy,  $\gamma$ -irradiation, immunotherapy, or cytokines primarily act by inducing apoptosis in target cells, and defects in apoptosis programs may cause resistance [2]. We previously found that BetA, a natural compound derived from white birch trees, induces apoptosis in tumor cells by directly triggering the mitochondrial pathway of apoptosis [11]. Importantly, BetA-induced apoptosis differs from apoptosis in response to treatment with anticancer agents such as doxorubicin because BetA-induced apoptosis does not involve p53 and is not associated with activation of ligand/receptor systems such as CD95 [11]. In the present study, we investigated whether or not activation of the mitochondrial pathway by BetA would modulate the sensitivity of tumor cells for anticancer drug-induced apoptosis. Here, for the first time, we provide evidence that combined treatment with BetA and anticancer drugs cooperated to induce apoptosis and to inhibit clonogenic survival of tumor cells.

Simultaneous treatment with BetA and anticancer drugs acted in concert to trigger loss of MMP and translocation of cytochrome *c* and Smac from mitochondria into the cytosol, resulting in activation of caspases, cleavage of endogenous caspase inhibitors such as XIAP, and cell

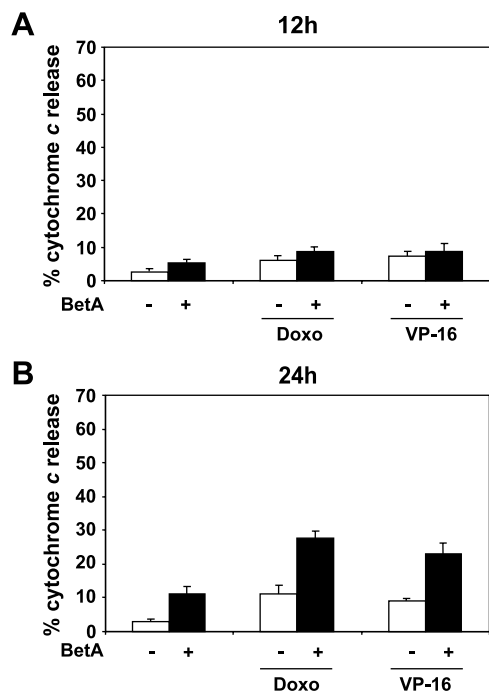


**Figure 6.** Cooperation of BetA and anticancer drugs to induce caspase activity. SHEP neuroblastoma cells were treated for 24 hours with 5  $\mu$ g/ml BetA and/or 0.2  $\mu$ g/ml doxorubicin and/or 3  $\mu$ g/ml VP16. Activity of caspase-3 (A), caspase-8 (B), or caspase-9 (C) was determined by enzymatic assay using fluorogenic caspase substrates as described in Materials and Methods section. X-fold increase in caspase activity compared to untreated controls is shown. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments.



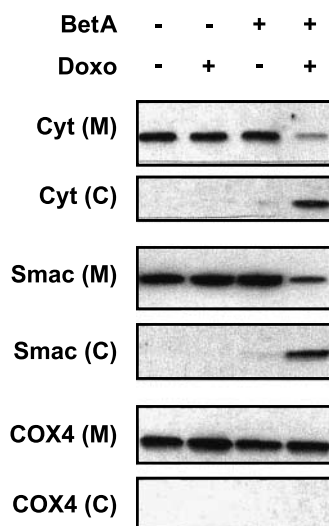
**Figure 7.** Cooperation of BetA and anticancer drugs to induce loss of MMP. SHEP neuroblastoma cells were treated for 12 hours (A) or 24 hours (B) with 5  $\mu$ g/ml BetA and/or 0.2  $\mu$ g/ml doxorubicin and/or 3  $\mu$ g/ml VP16. MMP was assessed by flow cytometry using the fluorescent dye, CMXRos. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments.

death. In contrast, single-agent treatment with suboptimal concentrations of BetA or various anticancer drugs induced only minimal mitochondrial alterations and minimal activation of caspases. Interestingly, loss of MMP and cytochrome *c* release on combined treatment with BetA and anticancer drugs involved caspase-dependent and also caspase-independent mechanisms. Although recent studies have implicated caspase-2 in the mitochondrial changes induced by cytotoxic agents in some cell lines [19], we found no detectable caspase-2 activation by Western blot analysis on combined treatment with BetA and anticancer drugs (data not shown). Because caspase-3 has been reported to feed back to damage mitochondria, enhanced caspase-3 activity on treatment with BetA and anticancer drugs may act back on mitochondria in an amplification loop. In addition, BetA may lower mitochondrially derived energy production and reduce repair following anticancer drug treatment, which may contribute to its sensitizing effect for anticancer agents. Importantly, combination treatment with BetA and anticancer drugs not simply delayed tumor growth, but inhibited clonogenic survival of tumor cells. Notably, the cooperation effect of BetA and anticancer drugs was found for a variety of cytotoxic compounds with different modes of action, including VP16, doxorubicin, cisplatin, Taxol, or actinomycin D. Thus, lowering the threshold for apoptosis induction by BetA can sensitize for apoptosis induced by various anticancer drugs, as most anticancer drugs predominantly act



**Figure 8.** Cooperation of BetA and anticancer drugs to induce cytochrome c release. SHEP neuroblastoma cells were treated for 12 hours (A) or 24 hours (B) with 5  $\mu$ g/ml BetA and/or 0.2  $\mu$ g/ml doxorubicin and/or 3  $\mu$ g/ml VP16. Release of cytochrome c from mitochondria was assessed by flow cytometry, staining cytochrome c in permeabilized cells as described in Materials and Methods section. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments.

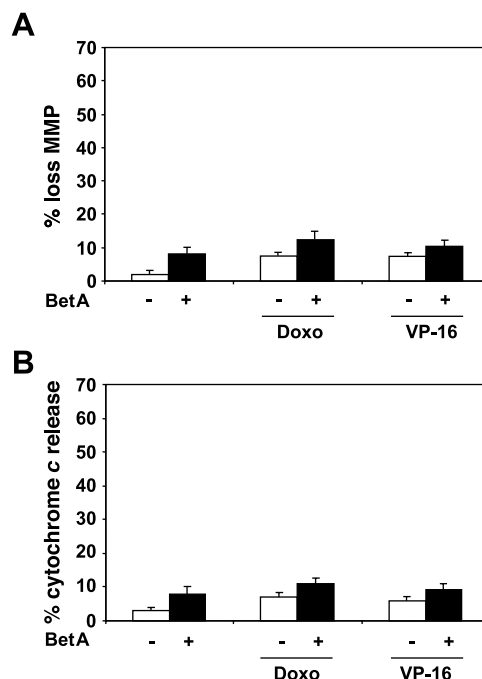
by triggering a common pathway of apoptosis in tumor cells. Importantly, the combination treatment with BetA and anticancer drugs cooperated to induce apoptosis in different tumor cell lines including *p53* mutant cells (Daoy



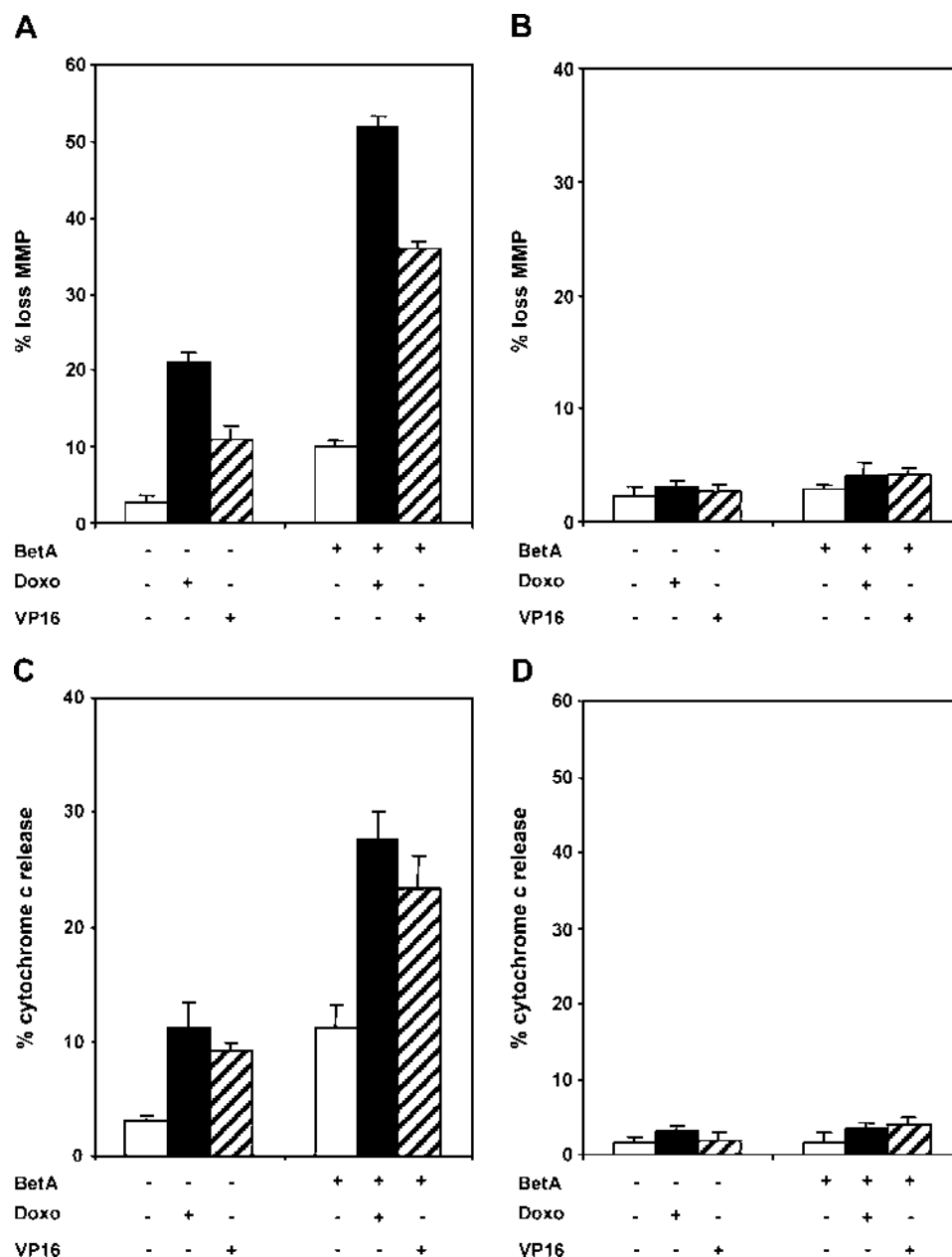
**Figure 9.** Cooperation of BetA and doxorubicin to induce release of mitochondrial proteins. SHEP neuroblastoma cells were treated for 24 hours with 5  $\mu$ g/ml BetA and/or 0.2  $\mu$ g/ml doxorubicin. Expression of Smac, cytochrome c, or COX4 in the mitochondrial (M) or cytosolic (C) fraction was assessed by Western blot analysis. A representative experiment out of three independent experiments is shown.

medulloblastoma cells). Also, BetA enhanced the sensitivity of primary tumor cells for anticancer drug-induced apoptosis. In contrast, BetA did not increase the sensitivity of untransformed human fibroblast for drug-induced apoptosis indicating some tumor specificity. To this end, we and other investigators have previously reported that BetA exerts selective cytotoxicity on tumor cell lines, but not on normal human cells [10,20]. BetA has been shown to trigger caspase-dependent apoptosis in a variety of tumor cells, in particular in neuroectodermal tumor cells, although caspase-independent cell death in response to BetA has also been described [8,9,11,21]. Also, BetA was found to act in concert with ionizing radiation, although the underlying mechanism has not exactly been defined [22].

By demonstrating that BetA can sensitize cancer cells for anticancer drug treatment, our findings may have several implications (e.g., for the proposed use of BetA in cancer therapy). Although pharmacokinetic data for BetA are limited at present, they indicate that pharmacologically active plasma levels may be achieved in rodents after systemic administration [23]. Because BetA significantly potentiated the antitumor activity of cytotoxic drugs, BetA may be used as a “sensitizer” in chemotherapy-based regimens to enhance the efficacy of anticancer agents for inhibition of tumor growth. Thus, the potential of BetA for anticancer therapy may particularly reside in its ability to



**Figure 10.** Effect of caspase inhibition on BetA and anticancer drug-induced loss of MMP and cytochrome c release. SHEP neuroblastoma cells were treated for 24 hours with 5  $\mu$ g/ml BetA and/or 0.2  $\mu$ g/ml doxorubicin and/or 3  $\mu$ g/ml VP16 in the presence of 50  $\mu$ M zVAD.fmk. In (A), MMP was assessed by flow cytometry using the fluorescent dye, CMXRos. In (B), release of cytochrome c from mitochondria was assessed by flow cytometry, staining cytochrome c in permeabilized cells as described in Materials and Methods section. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments.

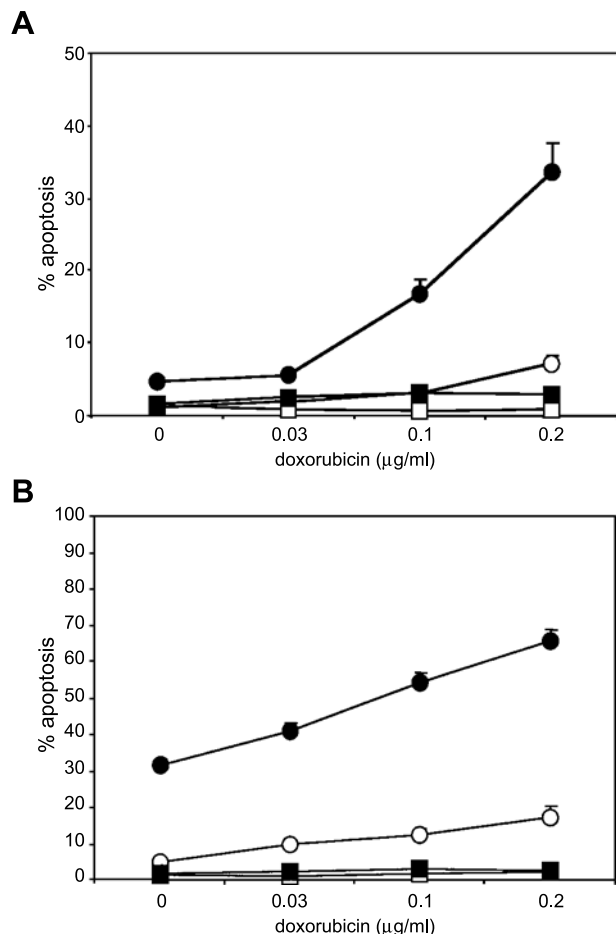


**Figure 11.** Effect of Bcl-2 overexpression on BetA and anticancer drug-induced mitochondrial perturbations. (A and B) Effect of Bcl-2 overexpression on BetA and anticancer drug-induced loss of MMP. SHEP neuroblastoma cells transfected with vector control (A) or Bcl-2 (B) were treated for 24 hours with 5  $\mu$ g/ml BetA and/or 0.2  $\mu$ g/ml doxorubicin and/or 3  $\mu$ g/ml VP16. MMP was assessed by flow cytometry using the fluorescent dye, CMXRos. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments. (C and D) Effect of Bcl-2 overexpression on BetA and drug-induced cytochrome c release. SHEP neuroblastoma cells transfected with vector control (C) or Bcl-2 (D) were treated for 24 hours with 5  $\mu$ g/ml BetA and/or 0.2  $\mu$ g/ml doxorubicin and/or 3  $\mu$ g/ml VP16. Release of cytochrome c from mitochondria was assessed by flow cytometry, staining cytochrome c in permeabilized cells as described in Materials and Methods section. Mean and SD of triplicates are shown; similar results were obtained from three independent experiments.

sensitize tumor cells for death induction or even to overcome resistance. The rationale for combination therapies is based on the concept that triggering tumor cell apoptosis through distinct compounds may help to amplify weaker death signals and ensure that apoptosis occurs within a certain time frame. Thus, combination treatments may prove to be advantageous in malignancies that still partially respond to either treatment alone because they potentially diminish the pool of tumor cells that give rise to an outgrowing resistant variant of the parental tumor and may

therefore constitute a potent strategy for bypassing resistance. Also, combination treatment using low doses of each agent may be advantageous in light of the fact that high concentrations found to be active *in vitro* may often not be achieved *in vivo*. Clinically, resistance to apoptosis is a major cause of primary or acquired nonresponsiveness of cancers leading to treatment failure. Thus, the combination of BetA and anticancer drugs may be a novel strategy to enhance the efficacy of chemotherapy-based regimens, which warrants further investigation.





**Figure 12.** Effect of Bcl-2 overexpression on BetA and doxorubicin-induced apoptosis. SHEP neuroblastoma cells transfected with vector control (circles) or Bcl-2 (squares) were treated for 24 hours (A) or 48 hours (B) with 0 to 0.2 µg/ml doxorubicin in the presence (closed symbols) or absence (open symbols) of 5 µg/ml BetA. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Note the different y-axis scaling in (A) and (B). Mean and SD of triplicates are shown; similar results were obtained from three independent experiments.

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